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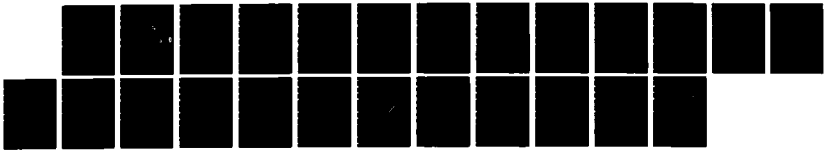

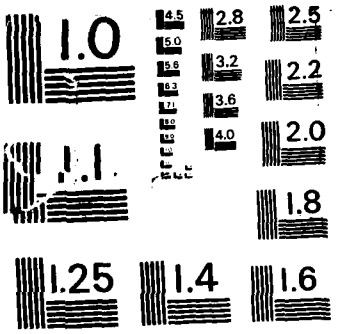

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ANNUAL REPORT

COY D. FITCH, M.D.

JANUARY 1985

Supported by
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

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Hemoglobin in glucose-6-phosphate dehydrogenase (G6PD)-deficient erythrocytes is abnormally vulnerable to oxidation, which may release ferriprotoporphyrin IX (FP), a potent lytic agent for erythrocytes and malaria parasites. To evaluate this possibility, we measured FP in G6PD-deficient erythrocytes. The FP content, as measured by spectrophotometry, was 13.3 nanomoles per gram of erythrocyte hemoglobin for G6PD-deficient erythrocyte membranes and 9.8 for normal erythrocyte membranes ($P < 0.05$). After incubation of erythrocytes with the oxidant drug, menadione, the values were 50.7 for G6PD-deficient and

20. ABSTRACT (continued)

35.8 for normal membranes ($P < 0.001$). Similar results were obtained with a radioligand binding assay using [^{14}C]-chloroquine and intact erythrocytes. Since FP which is accessible to bind chloroquine is available to lyse cells, we conclude that FP is available to mediate menadione-induced hemolysis and the toxicity of menadione for Plasmodium falciparum parasites growing in G6PD-deficient erythrocytes. We also propose that accumulation of FP may account for the resistance to malaria afforded by G6PD deficiency and for the destruction of senescent erythrocytes.

Because of the biological importance of the interaction of FP with cell membranes, we are evaluating the possibility that FP and certain antimalarial drugs act by altering membrane fluidity. To assess fluidity, 16-DOXYL-stearic acid was incorporated into washed erythrocyte membranes. FP and mefloquine, but not chloroquine, caused significant increases in fluidity as detected by increases in $2T_{\perp}'$, a parameter of the electron paramagnetic resonance (EPR) spectrum of membrane bound 16-DOXYL-stearic acid which is sensitive to changes in fluidity. Treatment of membranes from 1 ml of packed erythrocytes with 1 μmole of FP or 10 μmoles of mefloquine caused increases in $2T_{\perp}'$ from 24.27±0.12 G for control preparations to 24.66±0.08 G for FP and 25.02±0.14 G for mefloquine-treated preparations (mean ± SE for 4 experiments). These effects of FP and mefloquine correlate with the binding of these compounds to membrane phospholipids and with their hemolytic potential. We propose that an increase in membrane fluidity is a primary toxic effect of FP and of mefloquine.

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FOREWORD

Drs. Stephen K. Janney, J. Heinrich Joist, and H. James Armbrecht and Mr. Kairav Chevli participated in this work and they are coauthors of manuscripts that have been submitted for publication. Some of the present report is taken verbatim from these manuscripts. We adhered to the "Guide for Laboratory Animal Facilities and Care" as promulgated by the committee on the Guide for Laboratory Animal Resources, National Academy of Sciences, National Research Council.

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STATEMENT OF THE PROBLEM

Ferriprotoporphyrin IX is produced in erythrocytes by oxidative denaturation of hemoglobin, as occurs in Heinz body hemolytic anemias, and by proteolytic degradation of hemoglobin, as occurs in erythrocytes infected with malaria parasites. Since FP is lytic for malaria parasites and erythrocytes and since it has high affinity for certain antimalarial drugs, we have proposed that FP mediates (a) the chemotherapeutic action of chloroquine and perhaps mefloquine, (b) the protection against malaria provided by anemias which are characterized by hemoglobin denaturation, including sickle cell anemia and glucose-6-phosphate dehydrogenase (G6PD) deficiency, and (c) the hemolytic crises which occur in patients who have the types of anemia which protect against malaria. Our experimental work is intended to test these three hypotheses and to provide an understanding at the molecular level of the interactions between FP, drugs, and drug-FP complexes with cellular membranes.

BACKGROUND

Ferriprotoporphyrin IX (FP) is toxic for biological membranes, causing lysis of mouse and human erythrocytes (Chou and Fitch, 1980; Kirschner-Zilber et al., 1982), malaria parasites (Orjih et al., 1981; Fitch et al., 1982) and trypanosomes (Mesnick et al., 1977). Prior to hemolysis, erythrocytes exposed to FP exhibit massive potassium loss and increased osmotic fragility (Chou and Fitch, 1981). This toxicity is of interest because lytic amounts of FP may be produced when hemoglobin is degraded or oxidatively denatured. Indeed, FP is released in large quantities when malaria parasites degrade hemoglobin, and it serves as a receptor for chloroquine and related drugs in erythrocytes infected with malaria parasites (Chou et al., 1980; Fitch, 1983). The erythrocyte membrane may also be exposed to FP in patients with Heinz body hemolytic anemias during periods of oxidant stress (Fitch, 1983). In the present work we provide evidence that sufficient FP is available in G6PD-deficient erythrocytes to account for protection against malaria and for hemolytic episodes in G6PD-deficient patients exposed to oxidant drugs.

In comparison to mouse erythrocytes, human erythrocytes are relatively resistant to FP-induced hemolysis. For example, 5 μM FP causes extensive hemolysis of mouse erythrocytes, but only after a lag period and only when the incubation temperature is above 29° C (Chou and Fitch, 1980, 1981). Five μM FP causes little or no hemolysis of human erythrocytes, even when the incubation temperature is 37° C (Kirschner-Zilber et al., 1982). At a concentration of 10 μM FP or greater, however, hemolysis of human erythrocytes occurs with temperatures as low as 20° C, there is no lag before hemolysis commences, and the process is rapid, with a maximum value being approached within 20 or 30 minutes (Kirschner-Zilber et al., 1982). These differences between mouse and human erythrocytes raise the possibility that the structure of a biological membrane determines its degree of susceptibility to FP toxicity.

The possibility that FP toxicity may be mediated through an interaction with phospholipids is of particular interest because it is consistent with a final common pathway for the mode of action of chloroquine and mefloquine. The

high affinity of mefloquine for phospholipids (Chevli and Fitch, 1982) may permit it to directly alter membrane function. Chloroquine, on the other hand, has a low affinity for phospholipids (Chevli and Fitch, 1982), but chloroquine might have the same effect on biological membranes as mefloquine if a bridging molecule were available to connect chloroquine to membrane phospholipids. We have proposed that FP is such a bridging molecule. Accordingly, we have begun experiments to evaluate the response of erythrocytes to FP, the chloroquine-FP complex, mefloquine, and other amphipathic agents. The erythrocyte was used as a model in these initial studies. We also intend to study malaria parasites, using the information obtained from studies of erythrocytes to facilitate the design of experiments.

METHODS

After obtaining informed consent, blood was obtained by venipuncture from black, male patients with G6PD deficiency who were hospitalized at John Cochran Veterans' Administration Medical Center. Healthy adult male volunteers provided normal blood. A portion of the blood was used for cellulose acetate hemoglobin electrophoresis (Schneider, 1974) to exclude common hemoglobinopathies and for G6PD measurements (Beutler, 1966). The remainder was mixed with an equal volume of Tris medium (141 mM NaCl, 20 mM glucose, and 10 mM Tris, pH 7.4) containing 1 mg of heparin per ml. Erythrocytes were sedimented by centrifugation and washed 3 times with Tris medium to remove plasma and buffy coat before preparing suspensions for incubation with or without menadione and chloroquine, alone or in combination. The hemoglobin concentration of these erythrocyte suspensions was measured prior to incubation to serve as a common reference for expression of results.

To prepare membranes for spectrophotometric measurement of FP, washed erythrocytes were hemolyzed (Dodge, Mitchell, and Hanahan, 1963) using hypotonic Tris medium (5 mM NaCl, 10 mM Tris, pH 7.4) as the lysing medium. The membranes were recovered by centrifugation at 20,000 xg for 20 minutes at 4^{o} C and were washed 5 times with hypotonic Tris medium. The membranes were then dissolved in 2.5% SDS for measurement of FP content (Asakura *et al.*, 1977).

To prepare membranes for EPR studies, washed erythrocytes were lysed with 5 mM phosphate medium (pH 8.0), and the membranes were collected by centrifugation at 20,000 xg for 20 minutes at 4^{o} C and washed 4 times by resuspending in 30 volumes of 5 mM phosphate medium (pH 8). Following the last hypotonic wash, the membranes were resuspended in 30 volumes of phosphate-buffered saline (115 mM NaCl, 30 mM $Na_2 HPO_4$, pH 7.4), after which they were collected by centrifugation and stored in the refrigerator for 3 days or less prior to use.

The spin labels 16-DOXYL-stearic acid and 5-DOXYL-stearic acid were purchased from the Syva Company, Palo Alto, California. The 16-DOXYL-stearic acid tends to detect fluidity changes in the interior of the membrane whereas 5-DOXYL-stearic acid detects changes near the membrane surface. These spin labels were introduced into the membranes via the bovine serum albumin exchange method, as described by Hubbell and McConnell (1969) and employed by Armbrecht *et al.* (1982). After allowing 60 minutes at 24^{o} C for the exchange to occur, the mixture containing the spin label, bovine serum albumin, and erythrocyte membranes was centrifuged at 20,000 xg for 20 minutes at 4^{o} C, and the supernatant solution

containing the albumin and excess spin label was discarded. Thirty volumes of phosphate-buffered saline (pH 7.4) were added to each tube, and the membranes were suspended by gentle mixing using a pasteur pipette. Then the appropriate amount of FP and/or drug was added to the membrane suspension, mixed, and incubated at room temperature and pH 7.4 for 10 minutes, after which the membranes were collected by centrifugation at 20,000 xg for 20 minutes at 4° C. The supernatant solution was saved for spectrophotometric measurement of FP (Asakura et al., 1977), and the EPR spectrum of the pellet was recorded immediately. When FP and drug combinations were studied, FP was added first and incubated for 10 minutes at room temperature before the drug was added and the mixture was centrifuged to collect the membranes.

Stock solutions of 1 mM hemin (FP chloride) in 0.02 M NaOH and 10 mM mefloquine hydrochloride in 40% (v/v) ethanol were prepared freshly for each experiment. Chloroquine diphosphate was prepared in phosphate-buffered saline (pH 7.4). The EPR spectrum was not altered by small amounts of ethanol, such as were present in the experiments with mefloquine, or by storage of washed erythrocyte membranes in the refrigerator for as long as 3 days prior to study.

The EPR spectrum was obtained using a Varian E109E EPR spectrometer. For this purpose, an aliquot of the pellet was drawn into a 50 microliter glass capillary tube, which subsequently was placed inside an EPR quartz sample tube. The spectra were recorded at a field set of 3250 G, scan range of 100 G, modulation amplitude of 2 G, modulation frequency of 100 kHz, microwave power of 5 mW, and microwave frequency of 9130 MHz. The temperature was routinely maintained at 37±0.2° C by a Varian variable temperature unit and monitored by a copper-constantan thermocouple connected to a digital thermometer.

RESULTS

Under control conditions, in the absence of menadione, the FP content of isolated membranes was greater for G6PD-deficient than normal erythrocytes Fig. 1). The values were 13.3±2.8 (mean ± standard deviation) nanomoles of membrane ferriheme (FP)/gram of erythrocyte hemoglobin for G6PD-deficient and 9.8±2.4 for normal membranes ($P < 0.05$ as estimated by the Student's t test). After incubation of erythrocytes with 250 μM menadione, the values were 50.7±5.9 for G6PD-deficient membranes and 35.8±3.9 for normal membranes ($P < 0.001$). Thus, the G6PD-deficient erythrocytes contained more FP in their membranes and exhibited a greater increase in membrane FP when exposed to the oxidant stress of menadione. Similar results were obtained by measuring chloroquine binding to intact erythrocytes. Under control conditions, 69.5±8.6 nanomoles of chloroquine were bound per gram of hemoglobin in G6PD-deficient erythrocytes and 54.7±4.0 in normal erythrocytes ($P < 0.005$). After incubation of erythrocytes with 250 μM menadione the values were 87.5±6.1 for G6PD-deficient erythrocytes and 61.9±3.4 for normal erythrocytes ($P < 0.001$).

It should be recognized that the binding of chloroquine to intact erythrocytes includes both a high-affinity component attributable to FP (Chou and Fitch, 1981; Fitch et al., 1983; Chou, Chevli, and Fitch, 1980) and a low-

affinity component due to other erythrocytic constituents such as hemoglobin and methemoglobin (Chou, Chevli, and Fitch, 1980). Since treatment with menadione affects only the high-affinity component (Fitch et al., 1983), the magnitude of the difference between menadione-treated and control erythrocytes should be directly proportional to the amount of FP released from hemoglobin; in equilibrium dialysis experiments, one chloroquine molecule binds two molecules of FP (Chou, Chevli, and Fitch, 1980). The observed differences (after incubation with 250 μM menadione) were 17.9±5.9 nanomoles of chloroquine bound per gram of hemoglobin in G6PD-deficient and 7.2±2.6 in normal erythrocytes ($P < 0.005$). These results correlate well with the measured increases in FP content of erythrocyte membranes (Fig. 2).

Figure 3 shows typical spectra of the 16-DOXYL-stearic acid spin label in normal erythrocyte membranes, with and without addition of FP. FP consistently increased the distance between the two inner absorption lines ($2T_{\perp}'$ -1.6 G), indicating an increase in membrane fluidity (Hubbell and McConnell, 1971). In 4 separate experiments 1 μmole of FP increased $2T_{\perp}'$ from 24.27±0.12 to 24.66± 0.08 G (Table I). Figure 4 shows that the effect of FP is dose-dependent and provides an estimate of day-to-day variability in the technique. The data in Fig. 4 were obtained from 3 separate experiments using erythrocytes obtained on 3 different days from the same individual.

The effects of chloroquine and mefloquine alone and in combination with FP also were studied (Table I). Chloroquine alone had no effect on membrane fluidity, and chloroquine combined with FP had no greater effect than FP alone. By contrast mefloquine greatly increased $2T_{\perp}'$. The effects of FP and mefloquine were not additive, however, as $2T_{\perp}'$ in the presence of FP and mefloquine was similar to the value obtained with FP alone.

To put the changes induced by FP and mefloquine in perspective, the effect of ethanol was measured in some of the experiments. With 5% (v/v) ethanol in the incubation medium, which is known to increase membrane fluidity (Armbrecht et al., 1983), there was an increase in $2T_{\perp}'$ from 24.3±0.10 to 24.74±0.15 G in experiments using erythrocytes from 4 different subjects. Finally, to explore the site of action of FP in the membrane, parallel experiments were conducted with the 5-DOXYL-stearic acid probe, which monitors fluidity near the membrane surface. FP had no appreciable effect on the EPR spectra of membranes containing this probe.

DISCUSSION

The oxidant drug, menadione, was chosen for the present experiments for three major reasons. It can precipitate hemolytic episodes in patients with G6PD deficiency (Deutsch, 1966), it inhibits the development of P. falciparum malaria parasites selectively in G6PD-deficient erythrocytes (Friedman, 1979), and it can release sufficient FP intracellularly to cause hemolysis (Fitch et al., 1983). When FP is released intracellularly by menadione or added exogenously, it binds chloroquine with high affinity (dissociation constant of 10^{-6} to 10^{-7} M (Fitch et al., 1983; Chou, Chevli, and Fitch, 1980) and is available to lyse erythrocytes (Chou and Fitch, 1981; Fitch et al., 1983).

Since G6PD-deficient erythrocytes exposed to menadione contain an excess of FP in a form accessible to chloroquine, we conclude that the FP is available

to mediate the hemolysis induced by menadione in G6PD-deficient patients (Deutsch, 1966) and the selective toxicity of menadione for malaria parasites growing in G6PD-deficient erythrocytes (Friedman, 1979). Release of FP may also be involved in the pathogenesis of hemolytic episodes caused by other oxidant drugs in G6PD-deficient patients. In addition, G6PD-deficient erythrocytes may be particularly vulnerable to the oxidant stress produced by malaria parasites, which metabolize NADPH and generate H_2O_2 (Eckman and Eaton, 1979; Etkin and Eaton, 1974). The resultant release of FP could inhibit intraerythrocytic development of malaria parasites, thereby reducing parasitemia as has been observed (Luzzato, Usanga, and Reddy, 1969), and thus could explain genetic selection for G6PD deficiency.

Even in the absence of abnormal oxidant stress, enough FP may eventually accumulate within erythrocytes to cause their destruction. For example, the excess FP in G6PD-deficient erythrocytes under control conditions could account for the decreased erythrocyte survival observed in black individuals with G6PD deficiency (Brewer, Tarlov, and Kellermeyer, 1961) who are not in hemolytic crisis. Similarly, as there is evidence of a decrease in G6PD activity with increasing age of normal erythrocytes (Piomelli et al., 1968), we propose that FP accumulation may contribute to the destruction of senescent erythrocytes. We conclude, therefore, that an understanding of the interaction of FP, as well as certain antimalarial drugs, with cell membranes is essential.

The present results indicate that the interactions of FP and mefloquine with erythrocyte membranes significantly increase fluidity. Indeed, treatment of the membranes with FP or mefloquine caused as much increase in $2T_{\parallel}'$ as treatment with 5% ethanol. In the case of FP, it is possible that this effect reflects an interaction with the hydrophobic part of the membrane, since the changes were observed with the 16-DOXYL probe but not with the 5-DOXYL probe. Similar studies of mefloquine using the 5-DOXYL probe are not available.

The effects of FP and mefloquine on fluidity correlate with their ability to bind membrane phospholipids (Tipping, Ketterer, and Christodoulides, 1979; Shviro, Zilber, and Shaklai, 1982; Chevli and Fitch, 1982) and to cause hemolysis (Dutta et al., 1983). Similar concentrations of chloroquine have no apparent effect on fluidity, but chloroquine binds only weakly to membrane phospholipids (Chevli and Fitch, 1982) and is not hemolytic until millimolar concentrations are reached (Fitch, unpublished data). We propose, therefore, that a primary toxic effect of FP and mefloquine, but not chloroquine, is an increase in membrane fluidity. Manifestations of FP toxicity such as massive potassium leak, changes in osmotic fragility, and hemolysis (Chou and Fitch, 1981) thus would be secondary to an increase in membrane fluidity.

The ability of FP and mefloquine to increase membrane fluidity also may help to understand certain other previously inexplicable phenomena. For example, membranes of erythrocytes infected with P. berghei exhibit increased fluidity (Howard and Sawyer, 1980; Allred, Sterling, and Morse, 1983; Butler, Deslauriers, and Smith, 1984). Since P. berghei parasites degrade hemoglobin and accumulate large amounts of FP intracellularly (Slomianny, Prensier, and Charet, 1984) it is possible that the increase in fluidity is due to the presence of FP. Another previously unexplained phenomenon is the effectiveness of mefloquine in the treatment of chloroquine-resistant human malaria (Schmidt, 1973). Although chloroquine and mefloquine both are quinoline derivatives and share many properties in common (Sweeney, 1984), only mefloquine binds to phospholipids (Chevli

and Fitch, 1982) and increases membrane fluidity. This ability to directly affect the physical state of biological membranes may account for mefloquine's superiority in the treatment of chloroquine-resistant human malaria.

CONCLUSIONS

In addition to the extensive evidence that FP mediates the chemotherapeutic action of chloroquine as an antimalarial drug, there is now ample evidence to implicate FP as the toxic substance which inhibits malaria parasite growth and reproduction in erythrocytes harboring abnormalities which predispose to oxidative denaturation of hemoglobin. FP may be the lytic agent responsible for hemolytic crises in patients who have G6PD deficiency or other diseases which predispose to abnormal oxidative denaturation of hemoglobin.

FP increases the fluidity of biological membranes and may thereby cause lysis of malaria parasites and erythrocytes.

Mefloquine, an antimalarial drug with high affinity for phospholipids, increases membrane fluidity and this effect may help explain its chemotherapeutic superiority over chloroquine.

RECOMMENDATIONS

1. Studies of the effect of mefloquine, quinine, chloroquine, WR 180,409, WR 171,669, calcium, and detergents on FP binding to membranes and to liposomes prepared from purified phospholipids should be completed. The results of these studies should be correlated with the effects of these agents on FP-induced membrane damage.

2. Studies should be undertaken to determine whether or not mefloquine, FP, calcium and selected amphipathic agents exhibit the same interactions with the membranes of isolated malaria parasites as with the membranes of erythrocytes.

3. A comprehensive survey should be made to determine whether or not malaria parasites have potential receptors for chloroquine and mefloquine other than FP and phospholipids.

4. The molecular events distal to drug binding in malaria parasites should be fully described to complete our understanding of the mode of action of chloroquine, mefloquine, and related drugs.

5. A rapid test to predict cross resistance patterns of malaria parasites should be developed using pure FP and phospholipids instead of intact, infected erythrocytes.

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TABLE I

EFFECTS OF FP, CHLOROQUINE, AND MEFLOQUINE ON EPR SPECTRUM OF 16-DOXYL-STEARIC ACID IN ERYTHROCYTE MEMBRANES

ADDITION	$2T_{\perp}'$ (G)
None	24.27±0.12†
1.0 µmole FP‡	24.66±0.08*
10 µmole chloroquine	24.29±0.17
1.0 µmole FP + 10 µmole chloroquine	24.68±0.16*
10 µmole mefloquine	25.02±0.14*
1.0 µmole FP + 10 µmole mefloquine	24.65±0.17

† Means ± SE are shown for 4 preparations of erythrocyte membranes obtained from 4 different subjects.

‡ Each incubation mixture contained washed membranes prepared from one ml of packed erythrocytes.

* $P < 0.05$ when compared to the results of no addition by analysis of variance for repeated measures. Paired comparisons were made using Tukey's post hoc procedure (Winer, 1971).

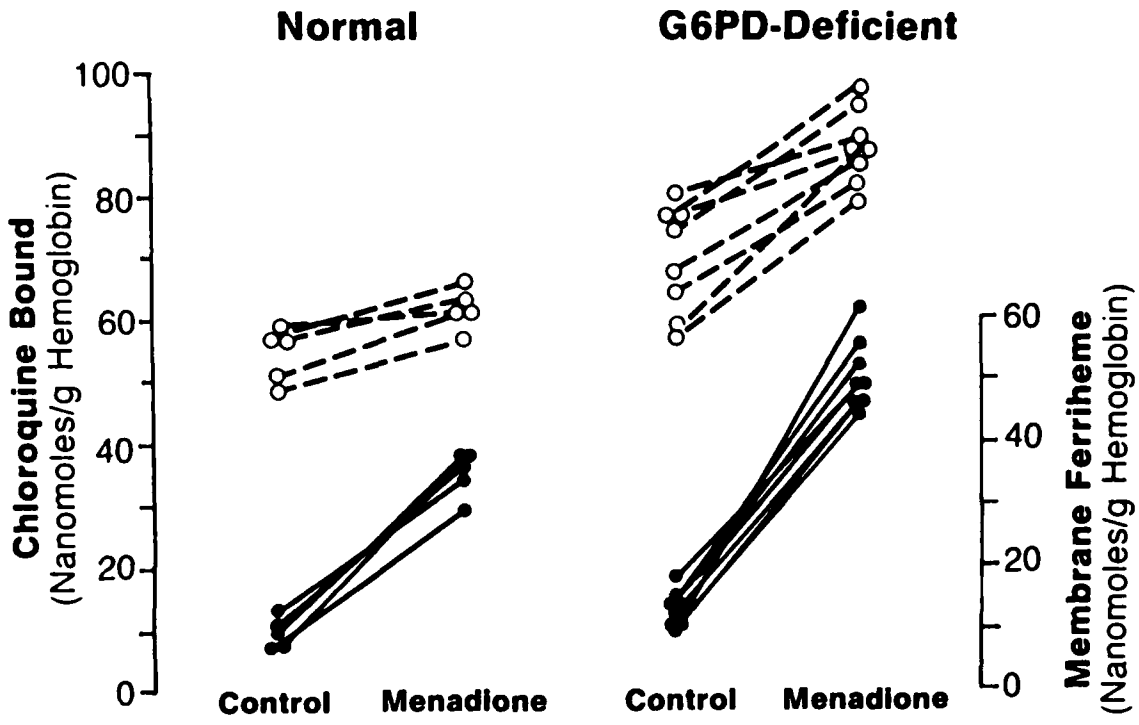

Fig. 1. Effect of menadione on FP (ferriheme) content of isolated erythrocyte membranes and on chloroquine binding to intact erythrocytes. Suspensions of erythrocytes (12.5% by volume) were incubated in Tris medium, pH 7.4, under room air for 60 minutes at 37° C in the absence (control) or presence of 250 μM menadione which was prepared as a stock suspension of 5 mM menadione in Tris medium containing 0.08% Tween 80 and sonicated immediately prior to addition to the incubation medium. In experiments in which chloroquine binding was measured, the initial concentration of chloroquine in the medium was 5 μM and ring-labeled 3-[^{14}C]-chloroquine (2.36 μCi/μmole; New England Nuclear Corporation) was used as a tracer (Fitch et al., 1983; Fitch, 1969). Values shown represent results of separate experiments with erythrocytes obtained from 5 normal subjects and 8 G6PD-deficient patients. Chloroquine bound to intact erythrocytes, o; FP content of isolated membranes, ●.

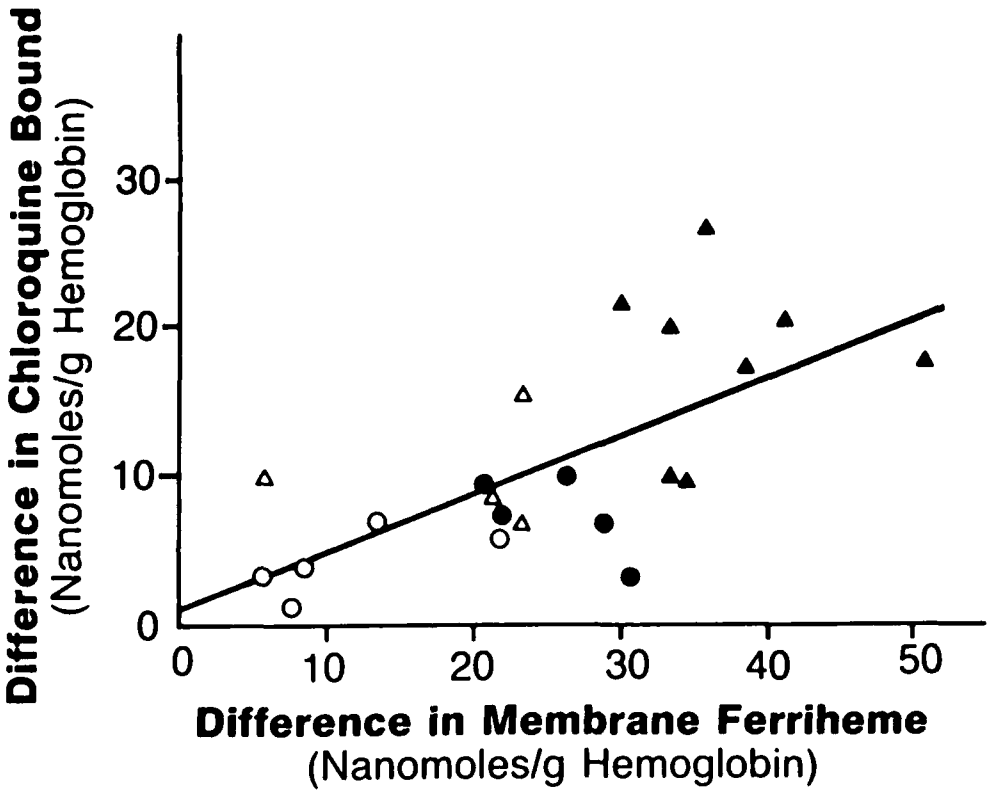

Fig. 2. Comparison of chloroquine binding to intact erythrocytes with FP (ferriheme) content of isolated erythrocyte membranes. Chloroquine binding (difference between the values in the presence and absence of menadione) is plotted on the ordinate; FP content (difference between the values in the presence and absence of menadione) is plotted on the abscissa. The experimental conditions were the same as described in Fig. 1 except that lower concentrations of menadione were used in some experiments. Normal erythrocytes with 125 μM menadione, o; G6PD-deficient erythrocytes with 125 μM menadione, Δ; normal erythrocytes with 250 μM menadione, ●; G6PD-deficient erythrocytes with 250 μM menadione, ▲. The regression line ($Y = 0.38\ X + 1.18$) is shown; the correlation coefficient is 0.666 ($P < .01$).

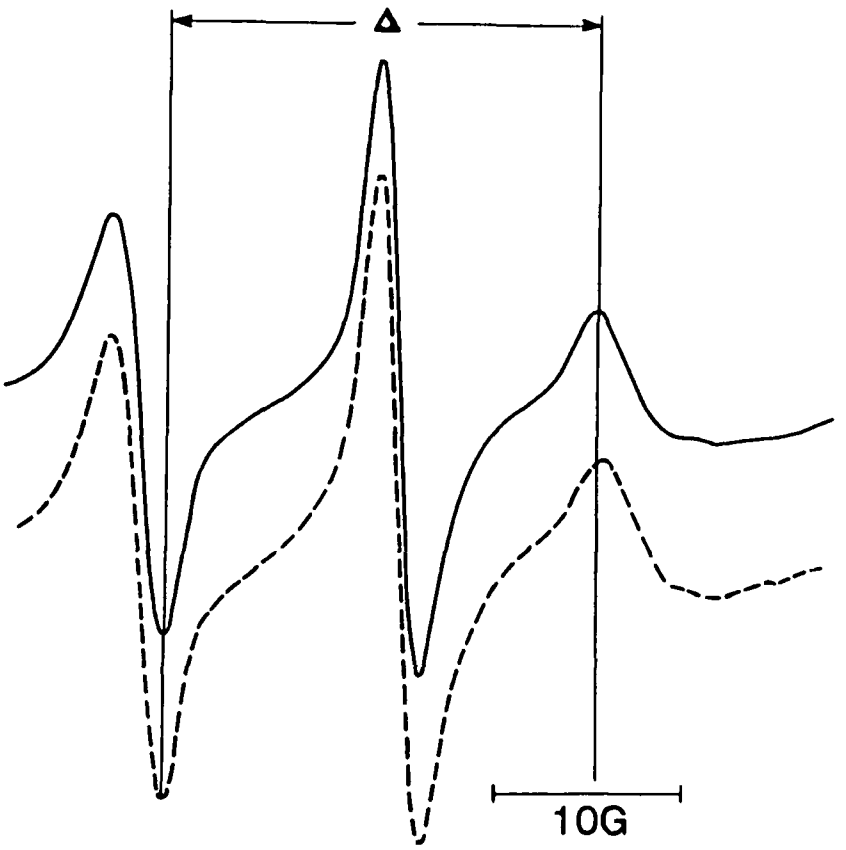

Fig. 3. First derivative EPR spectra of 16-DOXYL-stearic acid in erythrocyte membranes. 16-DOXYL-stearic acid was incorporated into washed membranes from 1 ml of packed erythrocytes as described in the text, after which the membranes were incubated at room temperature for 10 minutes in the presence or absence of 1 μmole of FP. After incubation and centrifugation approximately 20% of the FP remained in solution. The distance Δ was considered to be equal to $2T_{\perp}'$ minus 1.6 G (Hubbell and McConnell, 1971). The spectrum obtained in the presence of FP is shown by the dashed line. In this figure the troughs of the left absorption lines of the two spectra were aligned. Therefore, the effect of FP on $2T_{\perp}'$ is visible as a displacement of the peak of the right absorption line.

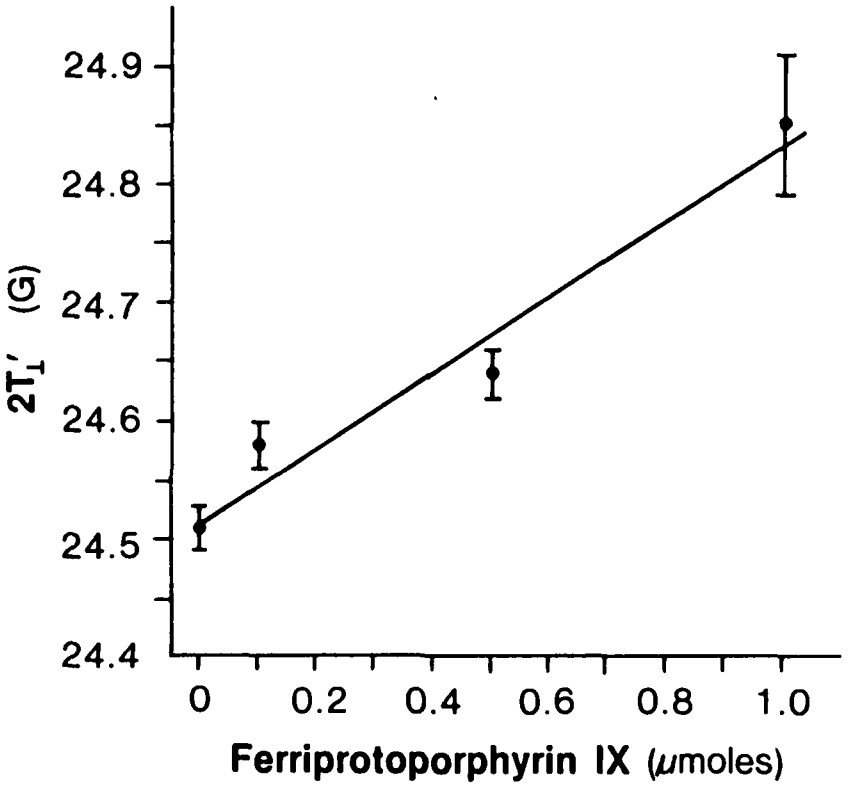

Fig. 4. Concentration dependence of the effect of FP. Washed erythrocyte membranes containing 16-DOXYL-stearic acid were treated with increasing amounts of FP as described in the legend to Fig. 3.

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